Fetuin A exacerbates palmitic acid induced podocyte death and can be attenuated by antagonizing Il-1β

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ABSTRACT

Diabetic nephropathy (DN) has become a leading cause of end stage renal disease (ESRD) in industrialized countries, and most affected patients have type 2 diabetes (T2D). Over the last years it has become evident that sterile inflammation plays a central role in the pathogenesis of DN. Accumulation of macrophages was demonstrated in humans and rodent models of DN, and inhibition of inflammatory cell recruitment into the kidney was shown to be protective in experimental DN. In addition to immune cells, intrinsic renal cells, such as podocytes and mesangial cells, can also secrete pro-inflammatory cytokines, which may contribute to the inflammatory process and aggravate DN.

The aim of the present study was to investigate whether palmitic acid, Fetuin-A or their combination elicit an inflammatory response involving toll-like receptor 4 (TLR4) in podocytes and whether this inflammatory pathway affects podocyte survival. Further, I aimed to investigate whether interleukin-1β (IL-1β) signaling can modulate podocyte death. Finally, I performed a pilot study in diabetic mice to investigate whether inhibition of IL-1β with a murinized anti-IL-1β antibody may attenuate albuminuria and/or surrogate markers of DN.

The present study uncovered that Fetuin-A or LPS exacerbate palmitic acid induced podocyte death, which is associated with a strong inflammatory response as indicated by the induction of monocyte chemoattractant protein-1 (MCP-1) and keratinocyte chemoattractant (KC). Moreover, blockage of TLR4 dramatically decreased MCP-1 and KC secretion and attenuated podocyte death induced by palmitic acid alone and/or Fetuin-A in combination with palmitic acid. In addition, inhibition of IL-1 signaling by anakinra, a recombinant human IL-1Ra, or a murinized anti-IL-1β antibody attenuated the inflammatory response elicited by Fetuin-A and palmitic acid and - similar to TLR4-inhibition - attenuated podocyte death.

In vivo, therapy of diabetic DBA/2J mice with an anti-IL-1β antibody prevented an increase in serum Fetuin-A concentrations and considerably decreased tumor necrosis factor alpha (TNFα) secretion in the urine, which is thought to correlate reciprocally with DN progression. In the short term, however, therapy with the anti-IL-1β antibody was not able to protect from albuminuria.

In summary, the results suggest that Fetuin-A leads to an inflammatory response in podocytes which exacerbates palmitic acid induced podocyte death. The data suggest also that TLR4 signaling plays a role as a modulator in the initiation and perturbation of the inflammatory process and leads to an exacerbation of palmitic acid induced podocyte death. In addition, this study implies a critical role for IL-1β signaling in palmitic acid induced podocyte death. *In vivo*, therapy of diabetic mice with an anti-IL-1β antibody favorably affected Fetuin-A levels in serum and urinary TNFα-levels, but in the short term a beneficial effect on albuminuria was absent, which gives the rational for prolonged studies to further test this new therapeutic approach.

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LIST OF ABBREVATIONS

Anak	Anakinra
ANG II	Angiotensin II
AP-1	Activator protein-1
BiP	Immunoglobulin heavy chain binding protein
BSA	Bovine serum albumin
CHOP	C/EBP homologous protein
DAG	Diglyceride
DMEM	Dulbecco’s modified eagle medium
DMSO	Dimethyl sulfoxide
DN	Diabetic nephropathy
DNA	Desoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
ER	Endoplasmic reticulum
ESRD	End-stage renal disease

FBS	Fetal bovine serum
FetA	Fetuin-A
FFA	Free fatty acid
GBM	Glomerular basement membrane
HEK	Human embryonic kidney
IL-1	Interleukin-1
IL-1β	Interleukin 1 β
IL-1Ra	IL-1 receptor antagonist
IL-12 p40	Interleukin-12 40 kDa
IFNγ	Interferon gamma
JNK	c-Jun NH_2-terminal kinase
KC	Keratinocyte chemoattractant
LPS	Lipopolysaccharide
MCP-1	Monocyte-chemoattractant protein-1
MUFA	Monounsaturated fatty acid
NF-κB	Nuclear Factor κB
PBS	Phosphate-buffered saline
PI	Propidium iodide
PPARα	Peroxisome proliferator-activated receptor α
SEAP	Secreted embryonic alkaline phosphatase
ROS	Reactive oxygen species
RPM	Revolutions per minutes
RPMI	Roswell Park Memorial Institute
SFAs	Saturated fatty acids
SD	Standard deviation
TG	Triglyceride
TGF-ß	Transforming growth factor ß
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor α
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes

1. INTRODUCTION

1.1. Diabetic Nephropathy

1.1.1. Epidemiology

Diabetic nephropathy is the most common cause end-stage renal disease (ESRD), it may affect one third of patients with diabetes mellitus (Molitch et al., 2004; "Type 2 diabetes in children and adolescents. American Diabetes Association," 2000). In many parts of the word, its incidence increased alarmingly over the last decades, which is related to the increase in obesity and type 2 diabetes (T2D). A further substantial increase is expected by 2050 (Reutens & Atkins, 2011). In some parts of the word, e.g. in the U.S., no further increase of incident DN was observed although its prevalence is still increasing (Burrows, Li, & Geiss, 2010). This finding suggests that strategies including early diagnosis, early initiation of therapy, and the use of more effective renoprotective medications are efficacious.

More recently, T2D - once thought to be a metabolic disorder of adulthood - has become increasingly more frequent in obese adolescents (Pinhas-Hamiel & Zeitler, 2005) and raised from less than 3% of all cases of new-onset diabetes in the 80s and 90s to 45% in adolescents by the end of the last century ("Type 2 diabetes in children and adolescents. American Diabetes Association," 2000).

1.1.2. Overview of factors contributing to the progression of diabetic nephropathy

There are several factors known to be imbalanced in the diabetic condition and which are thought contribute to the pathogenesis of DN including hyperglycemia (Susztak & Bottinger, 2006), impaired insulin signaling (Madhusudhan et al., 2015), advanced glycation end products (Chuang, Yu, Fang, Uribarri, & He, 2007) activation of cytokines (Lim & Tesch, 2012), and dyslipidemia (Sieber & Jehle, 2014). There is no single driving force that leads to DN, but a combination of factors that drive progression of DN. In diabetic patients, tissues including the liver, adipose tissue, and skeletal muscle are affected by impaired insulin signal (Wilcox, 2005). Insulin resistance and relative or absolute lack of insulin (insulinopenia) lead to hyperglycemia (Rosival, 2007) and release of FFAs into the circulation which may exacerbate insulin resistance (Sieber & Jehle, 2014). FFAs can accumulate in the glomeruli (Bobulescu, 2010) and may cause cellular damage in a process named lipotoxicity (Schaffer, 2003). Chronic low grade inflammation critically contributes to the pathogenesis of T2D (Donath & Shoelson, 2011) and accumulating evidence suggests that inflammation plays also a significant role in the pathogenesis of DN (Navarro & Mora, 2005).

1.1.3. Podocytes in the pathogenesis of DN

The kidney glomerular filter is a charged-selective barrier that allows blood filtration without loss of large proteins into the urine. The filtration unit in the kidney has three layers: fenestrated endothelial cells, the glomerular basement membrane (GBM), and podocytes. Defects in any of the three layers in the glomeruli can lead to proteinuria. The prominent role of podocytes for the function of the glomerular filter is illustrated by the identification of single human gene defects important for podocyte function, which lead to heavy proteinuria and the so called nephrotic syndrome (proteinuria over 3g per day, oedema, hypoalbuminemia) (Greka & Mundel, 2012).

Moreover, podocytes critically determine the biophysical characteristics of the GBM and are important to counteract the hydrostatic pressure from

the glomerular capillaries (Endlich & Endlich, 2006).

A

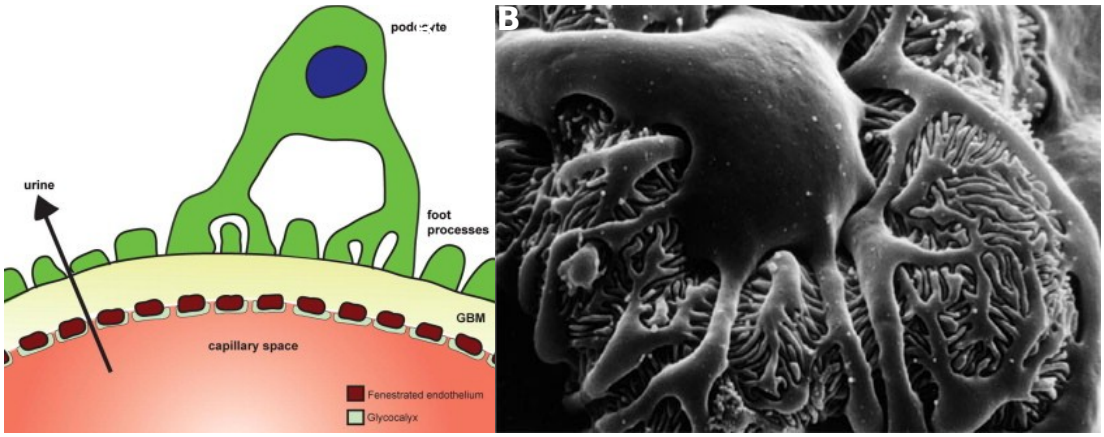

Figure 1: Structure of the glomerular filtration barrier. A) Schematic picture of the glomerular filtration barrier consisting of fenestrated endothelium cells, the glomerular basement membrane (GBM), and podocytes with their interdigitating foot process (Image from (Patrakka & Tryggvason, 2010). B) Image taken using a scanning electron microscope of a podocyte wrapped around a glomerular capillary (Image from (Smoyer & Mundel, 1998).

In response to injury, podocytes may undergo a number of different fates that include proliferation, de-differentiation, or apoptosis. Podocytes are highly differentiated epithelial cells with low capability to undergo cell division (Kriz, 2003; Reidy & Kaskel, 2007).
Damage and loss of podocytes critically contributes to DN (Wolf, Chen, & Ziyadeh, 2005). It is well reported in T1D and T2D patients that they have a reduced number and density of podocytes (Dalla Vestra et al., 2003; Pagtalunan et al., 1997; Steffes, Schmidt, McCrery, & Basgen, 2001; White & Bilous, 2000, 2004). Importantly, podocyte loss directly correlates with proteinuria(White & Bilous, 2004). Even more, a study performed in Pima Indians with T2D suggests that a reduced number of podocytes per glomerulus predicts progressive kidney disease (Meyer, Bennett, & Nelson, 1999).

1.1.4. Factors contributing to apoptosis of podocytes in DN

It has become clear that hyperglycemia alone is neither the only contributing factor nor sufficient to cause diabetic kidney disease and podocyte death. An increasing number of hemodynamic, metabolic,

inflammatory, structural, and genetic factors have been identified in the disease process (Dronavalli, Duka, & Bakris, 2008).
In vitro, high glucose concentrations have been reported to induce apoptosis of podocytes, and increased ROS (reactive oxygen species) levels have been shown to be important mediators of glucotoxicity (Eid et al., 2009; Kanwar et al., 1996; Susztak & Bottinger, 2006). ROS do not result from hyperglycemia only, but are also stimulated by angiotensin II (ANG II). Transforming growth factor (TGF-β) is another important factor leading to podocyte death (Schiffer, Daxenberger, Meyer, & Meyer, 2001; T. Wada, Pippin, Terada, & Shankland, 2005). In the diabetic milieu, TGF-β is induced by many factors including ROS, ANG II, and advanced glycation end products (Ziyadeh, 2004). Administration of neutralizing anti-TGF-β antibodies in db/db mice was found to prevent mesangial matrix expansion and to protect from a decline in kidney function (Ziyadeh et al., 2000).
In the following I will introduce in more detail the role dysregulated lipid metabolism as well as inflammation in the pathogenesis of podocyte damage and DN.

1.2. Lipotoxicity in the pathogenesis of DN

Elevated FFAs in the circulation lead to lipid accumulation in non-adipose tissues including the pancreas, heart, liver, and the kidneys. Excessive lipid droplets can be found in different renal cell types including podocytes (de Vries et al., 2014; Herman-Edelstein, Scherzer, Tobar, Levi, & Gafter, 2014). Lipid deposition causes cell dysfunction and death. This process is term lipotoxicity (Brookheart, Michel, & Schaffer, 2009).
Disturbed fatty acid metabolism and elevated FFAs are critical determinants of lipotoxicity (Brookheart et al., 2009). Up to 80% of the plasma FFAs consist of the saturated palmitic acid (C16:0) and stearic acid (C18:0) as well as the monounsaturated oleic acid (C18:1) (Hagenfeldt, Wahren, Pernow, & Räf, 1972), though quantification of FFAs in circulation is controversial due to their tendency to aggregate. Adipocytes of obese individuals can become defective in FFA uptake, which adds to elevated

FFA-levels and favors ectopic fat deposition (McQuaid et al., 2011). This is of interest as plasma FFA composition partially reflects dietary fatty acids (Fielding et al., 1996) and saturated FFAs (SFAs) and monounsaturated FFAs (MUFAs) have distinct effects on cell metabolism and function. The toxicity of FFAs has been attributed mainly to SFAs whereas MUFAs exert beneficial and cytoprotective effects (Brookheart et al., 2009; Nolan & Larter, 2009).

Dysregulared fatty acid metabolism is thought to play an important role in the development of DN and in kidney diseases related to metabolically unhealthy obesity (Sieber & Jehle, 2014). Diabetic patients present an altered serum lipid profile characterized by elevated triglyceride (TG) concentrations beginning in the earliest stages of microalbuminuria (Murea et al., 2010). Dyslipidemia may affect the kidney directly by causing deleterious renal lipid accumulation and indirectly through systemic inflammation, oxidative stress, vascular injury, and changes in hormones and other signaling molecules with renal action (Bobulescu, 2010).

1.2.1. Effects of FFAs and of their metabolism on podocyte survival

Our lab has shown that palmitic acid increases podocyte death in a dose- and time-dependent fashion. Palmitic acid induces podocyte ER (endoplasmatic reticulum) stress leading to an unfolded protein response as reflected by the induction of the ER chaperone immunoglobulin heavy chain binding protein (BiP), and proapoptotic C/EBP homologous protein (CHOP) transcription factor, and gene silencing of CHOP protects at least in part from palmitic acid induced podocyte death (Sieber et al., 2010). Similar to other cell types including pancreatic β-cells (Maedler, Oberholzer, Bucher, Spinas, & Donath, 2003) and human mesangial cells (Mishra & Simonson, 2005) MUFAs protect podocytes from palmitic acid induced cell death (Sieber et al., 2010). Tracing studies with tritium-labeled palmitic acid demonstrated that oleic acid leads to preferential incorporation of palmitic acid derived metabolites into TGs (Sieber et al., 2013). In other words, oleic acid may reduce palmitic acid-induced

podocyte death by shifting palmitic acid and its toxic metabolites to “safe” TG pools. Furthermore, oleic acid stimulates β-oxidation of palmitic acid and may be beneficial simply by reducing the levels of palmitic acid and its toxic metabolites (Kampe, Sieber, Orellana, Mundel, & Jehle, 2014).

1.3. Inflammation in the pathogenesis of DN

Chronic low grade inflammation is thought to be one of the most important underlying mechanisms in the pathogenesis of T2D (Donath & Shoelson, 2011). Similarly, accumulating evidence suggests that inflammation plays a significant role in the pathogenesis of DN (Navarro & Mora, 2005). Interestingly, it is suggested that there is a reciprocal relationship between the metabolic syndrome and chronic inflammation (Hotamisligil, 2006). Accumulation of macrophages was demonstrated in humans and rodent models of DN (F. Chow, Ozols, Nikolic-Paterson, Atkins, & Tesch, 2004; Nguyen et al., 2006) and inhibition of inflammatory cell recruitment into the kidney was shown to be protective in experimental DN (Awad et al., 2011; F. Y. Chow, Nikolic-Paterson, Ozols, Atkins, & Tesch, 2005). In addition to immune cells, intrinsic renal cells such as podocytes and mesangial cells can also secrete pro-inflammatory cytokines, which may contribute to the inflammatory process and aggravate DN (Sayyed et al., 2009; Tesch et al., 1997)

1.3.1. Toll-like receptors and DN

Toll-like receptors (TLRs) are considered to play a key role in the initiation and perturbation of the inflammatory process underlying diabetes and its complications (Lin & Tang, 2014). TLRs recognize pathogen-associated molecular patterns such as lipopolysaccharide (LPS), and viral or bacterial nucleic acids (Akira, Takeda, & Kaisho, 2001). Activation of TLRs leads to recruitment of adaptor proteins, which subsequently trigger downstream signaling cascades resulting in activation of nuclear factor-κB (NF-κB) (Akira et al., 2001). The transcription factor NF-κB induces a wide range of cytokines including interleukin-1β (IL-1β) (Hiscott et al., 1993) and monocyte-chemoattractant protein-1 (MCP-1) (Ueda et al., 1994).

Importantly, it has been shown that TLRs are not activated by pathogen-associated molecular patterns only but also by endogenous danger signals released during tissue injury or metabolic stress (Akira et al., 2001).
T2D is characterized by hyperglycemia and dyslipidemia with increased levels of free fatty acids (FFAs) (Randle, Garland, Hales, & Newsholme, 1963). Over the last years growing data indicate that FFAs can induce the production of pro-inflammatory cytokines through activation of TLR4 and/or TLR2 (Boni-Schnetzler et al., 2009). However, how FFAs activate TLRs is a subject of debate and current evidence suggests that FFAs do not directly bind to TLR4 (Erridge & Samani, 2009). Recently, a model was suggested with Fetuin-A (FetA) as an endogenous ligand and potential molecular linker for FFAs to TLR4 (Pal et al., 2012).

1.3.2. Fetuin-A

Fetuin-A (FetA) is a liver-derived, abundant plasma protein which is involved in multiple biological functions. The function of FetA is best studied as a carrier protein for otherwise insoluble mineral and as an inhibitor of ectopic calcium deposition (Jahnen-Dechent, Heiss, Schafer, & Ketteler, 2011). In addition and already dating back to the 90's, FetA is known as a major carrier protein of FFAs in the circulation (Cayatte, Kumbla, & Subbiah, 1990). Urinary FetA is also a new candidate marker to predict progression of DN (Inoue et al., 2013). The first study which suggests that FetA might be the missing linker of FFAs to TLR4 was published in Nature Medicine by Bhattacharya and co-authors (Pal et al., 2012).

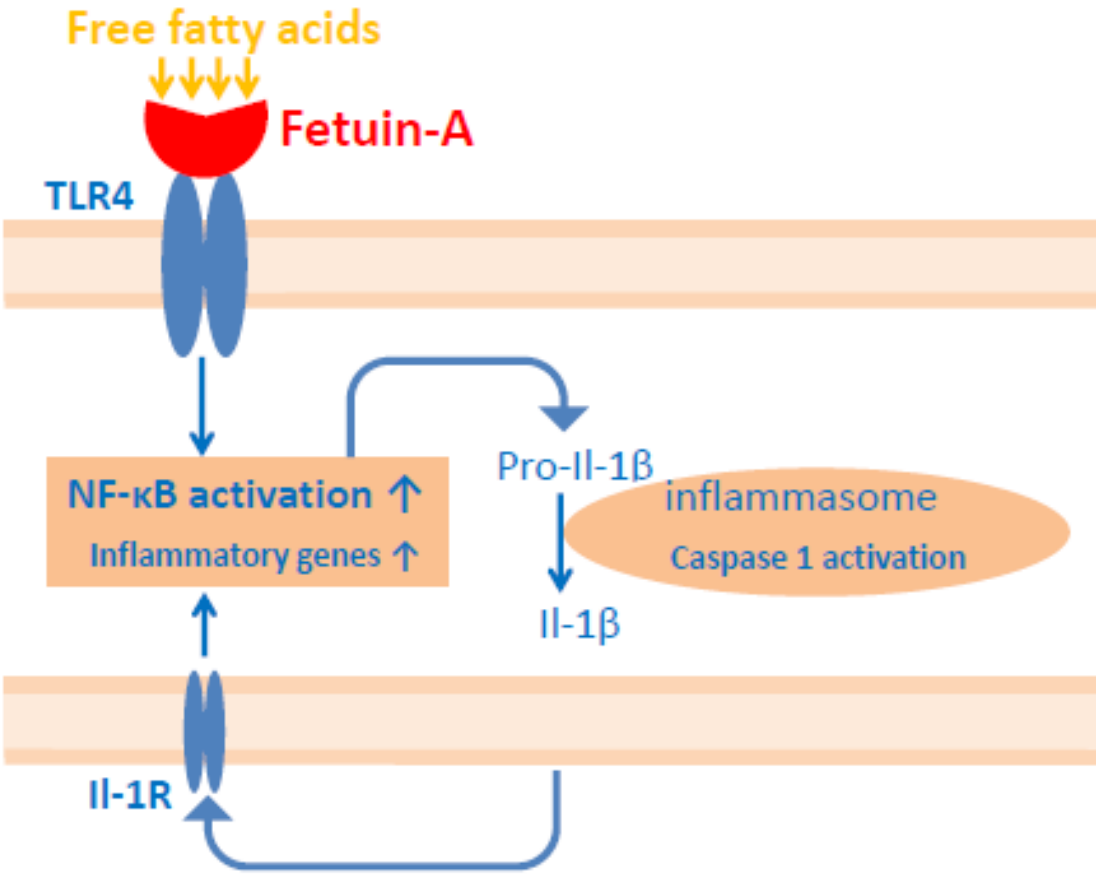

Figure 2: Model for Fetuin-A linking FFAs to TLR4 signaling. Fetuin-A is proposed to be the link between FFAs and the TLR4 pathway. TLR4 signaling leads to the activation of NF-κB, which upregulates the transcription of inflammatory genes including pro-Il-1β. Pro-Il-1β is processed by the inflammasome associated caspase-1. In turn, Il-1β sustains autocrine (and paracrine) activation of local cells including podocytes which leads to a chronic inflammatory response in the kidney and promotes chronic kidney disease.

1.3.3. Interleukin-1β

Interleukin-1β is a master regulator of inflammation in various tissues (Dinarello, 2009). Intrinsic renal cells including podocytes are major sources of IL-1β in experimental models of glomerulonephritis (Niemir et al., 1997).

The potential role of IL-1β in the pathogenesis of DN dates back to 1996 when it was reported that an allele of the IL-1 receptor antagonist (IL-1Ra), which finally leads to increased IL-1 signaling, is associated with DN (Blakemore et al., 1996). IL-1 β induces also other chemokines, which in turn attracts macrophages (Ehses et al., 2009). The vicious cycle is further amplified as IL-1 β can induce lipoprotein lipase which may further increase local and systemic concentrations of free fatty acids (Dinarello, 2011). More recently, it was found that IL-1β is elevated in plasma and in renal cortex extracts at the onset of DN, and anakinra, a recombinant human IL-1Ra, can prevent or even reverse DN in different mouse models (Shahzad et al., 2015).

1.4. Aim of the study

Increasing evidence suggests that in the pathogenesis of DN podocyte injury and loss are critical events and determine disease progression and precede proteinuria. Several factors of the diabetic milieu are known to impair function and survival of podocytes. Although lipid accumulation is a well know feature of DN, only recently the potentially important role of FFAs and FFA metabolism in this process were acknowledged.

Over the last years our laboratory systematically analyzed the effects of FFAs and FFA metabolism on podocyte survival. The objective of the present study was to investigate whether FetA or TLR4 activation by LPS exacerbates palmitic acid induced podocytes death and whether this may involve inflammatory pathways with a special focus on IL-1β. In a preliminary study *in vivo*, I investigated the effect of an anti-IL-1β antibody on insulinopenic DBA/2J mice on a high fat diet.

2. MATERIAL AND METHODS

2.1. Cell culture & Reagents

Podocytes were cultured following the protocol described by Mundel et al. (Shankland, Pippin, Reiser, & Mundel, 2007). Conditionally immortalized mouse podocytes obtained from Peter Mundel carry a thermosensitive variant of the SV 40 T antigen as a transgene (Shankland et al., 2007). Podocytes were cultured in RPMI-1640 (#21875, Invitrogen), enriched with 10% heat-inactivated FBS (#10270, Invitrogen) and 100 U/ml penicillin/streptomycin (#15140, Invitrogen). Podocytes proliferated under growth-permissive conditions at 33°C with IFN-gamma (IFNγ) (#CTK-358-2PS, MoBiTec GmbH, Germany), and differentiate for 11 days without IFN-γ at 37°C (Sieber et al., 2013). Podocytes having a passage number from 4 to 14 were employed for experiments. Cell culture plates and flasks used in all assays were coated with type I collagen (BD Biosciences). For freezing cells complete culture medium was employed supplemented with 8% (v/v) dimethylsulfoxide (D2438, Sigma).

Table 1: Agonist, inhibitors and used compounds

Substance	Catalog number	Supplier	Action	Working concentration
LPS	L2630	Sigma	TLR4 activator	10 pg to 1 µg/ml
Fetuin-A, Bovine	Non commercialized product	Provided by Prof. Jahnen-Dechent, Aachen University	Carrier protein, TLR4 activator, Insulin inhibitor	200 µg/ml, < 0.06 EU/ml tested with Endosafe ultrasensitive PTS, Charles River.
Fetuin-A, murine	Non commercialized product	Donated by Prof. Bhattacharya. Visva-Bharati University	Carrier protein, TLR4 activator, Insulin inhibitor	200 – 350 µg/ml, <0.005 EU/ml tested with EndoTrap® HD, Hyglos GmbH.
Anakinra	Kineret®	SOBI	IL-1 receptor antagonist	1 µg/ml
TAK-242	tlrl-cli95	Invivogen	TLR4 signaling inhibitor	0.1 µg/ml
Anti IL-1 β Ab	Non commercialized product	Novartis	IL-1β signaling inhibitor	3.3 µg/ml
IL-1 β	I5271	Sigma	Inflammatory citokine	5 ng/ml

2.2. **Free fatty acids preparation**

Palmitic acid 10 mM (P9767, Sigma) was dissolved overnight in 11% essential fatty-acid free low endotoxins BSA (A8806, Sigma) in RPMI [-]D-glucose(11879-020, LuBioscience), shaken at 37°C under N2-atmosphere for 20 min, sonicated for 10 min, sterile filtered, and stored at −20°C. The molar ratio of fatty acid to BSA was 6:1. Before use, the complexes were heated at 37°C for 15 min followed by dilution in media. BSA used for control experiments was prepared and handled exactly the same as BSA

complex to FFAs (Boni-Schnetzler et al., 2009). The effective free fatty acid concentrations were measured with a commercially available kit (Wako).

2.3. **Removal and measurement of endotoxins**

Fatty acid preparations, bovine and murine FetA (Pal et al., 2012), were cleaned of endotoxins with EndoTrap® HD (800063, Hyglos GmbH). Endotrap is supplied in prepacked columns that contain an affinity matrix for the efficient removal of endotoxins from aqueous solutions. Endotrap columns can be used more than 10 times. Our preparations were passed through the columns 4 times. Each round of application should yield a two log reduction of endotoxins. According to manufacturer, loss of proteins resulting from binding to endotrap is extremely low, less than 5%. Concentration of solutions after and before cleaning the samples was determined by DC Protein Assay (Bio-Rad).
The endotoxin concentration was subsequently measured with ToxinSensor Chromogenic LAL Endotoxin Assay Kit (L00350C, GenScript) following manufacturer instructions. Limulus Amebocyte Lysate (LAL) is an extract of circulating blood cells of the American Horseshoe crab. Endotoxins cause amebocytes clotting and trigger the turbidity. This enzymatic reaction leads to a colorimetric reaction that allows the quantification of endotoxin. All reactions were performed strictly in a constant temperature of 37°C in the heating block. Tips and plasticware used were endotoxin-free. The level of endotoxins was confirmed to be <0.005 EU/ml.

2.4. **Toll-like receptor activation assay**

TLR4 activation assay was done by using HEK-Blue hTLR4 reporter cells (Invivogen). Cells were generated by co-transfecting HEK293 cells with hTLR4 gene, MD-2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene. SEAP is expressed under the control of the IL-12 p40 minimal promoter fused to five NF-κB and AP-1 (Activator protein-1) binding sites.
Cells were cultured in 75 cm^2 flasks with DMEM (#41965, Invitrogen) media including 4.5 g/l glucose, 2-4 mM L-glutamine, 10% FBS,

penicillin/streptomycin, and Normocin according to manufacturer instructions. Experiments were done in 96-well plates with palmitic acid in the presence of FetA, ranging from 0 µg/ml to 150 µg/ml or LPS, ranging from 10 pg/ml to 10^4pg/ml for 6h. Secreted SEAP turned the cell culture media in purple/blue color, which was quantified with Synergy H1 hybrid reader (BioTek).

2.5. **Apoptosis assay**

Experiments were performed in six-well plates, cultured in RPMI-1640, with 0.2% heat-inactivated FBS. Supernatants were collected, cells were trypsinized and harvested by rinsing with complete media (DMEM/10% FBS/penicillin/streptomycin) and kept on ice. Cells were centrifuged at 1400 rpm at 4°C for 5 minutes, and pellet was washed twice with 500 µl cold PBS. Cells were resuspended in 100 µl annexin-binding buffer (10 mM Hepes/140 mM NaCl/2.5 mM CaCl2, pH 7.4) and incubated for 15 min in dark at room temperature with 1:25 Alexa Fluor 647 annexin V conjugate (A23204, life technologies). The sample final volume was 500 µl by adding annexin-binding buffer. Before analyzing the samples 0.5 µg propidium iodide (P3566, Invitrogen) was added to identify necrotic cells (Sieber et al., 2013). 20,000 events were acquired by Accuri C6 flow cytometer (BD Bioscience, Mountain View, CA). Data from flow cytometry was analyzed by the software program FLOWJO (Tree Star, Ashland, OR). Annexin V-positive/PI-negative podocytes were considered apoptotic, while double positive cells were considered late apoptotic/necrotic cells.

2.6. **Measurement of cytokines and chemokines**

Differentiated podocytes cultured in 75 cm^2 flasks were re-seeded in 96-well plates for 24h, at 6000 cells/well. Before the experiment, cells were synchronized in serum starvation medium (RPMI-1640, with 0.2% heat-inactivated FBS and 5 mM/l glucose) for 16 hrs and for the experiments same medium was maintained. After the experiment, supernatants were centrifuged at 11000 rpm for 3 minutes and were diluted 1:20 for the detection of MCP1. For the detection of KC no dilution was done. MCP1 and KC expression was quantified using Duoset ELISA kit according to

manufacturer instructions (DY479 for MCP-1; DY453 for KC, R&D Systems purchased from Zug, Switzerland).

2.7. Animal experimentation

For the animal experiments, 8-week-old DBA/2J (DBA) male mice were purchased from Charles River (Sulzfeld, Germany) and for experiments with C57BL6/N (B6), 4 week old male mice were purchased from Janvier labs, France. Mice were maintained in 12h-light/12h-dark cycle and provided food and water ad libitum. For DBA experiments, mice were randomly divided into four groups: group 1: control (Chow diet) + saline (n=6), group 2: control (Chow diet) + anti IL-1β Ab (n=6); group 3: STZ (HFD) + saline (n=6); group 4: STZ (HFD)+ anti IL-1β Ab (n=6).
For experiments with B6 mice, animals were divided into four groups: group1: control +saline (n=6), group 2: control + anti IL-1β Ab (n=6), group 3: STZ + saline (n=6), group 4: STZ+ anti IL-1β Ab (n=6). All experiments with mice were performed following the Swiss veterinary law and institutional guidelines.

2.7.1. Induction of diabetes and anti IL-1β treatment

To induce diabetes, DBA mice were injected with 40 mg/kg of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) intraperitoneally for five days, freshly prepared in 50 mmol/l sodium citrate buffer at pH 4.5. Control mice were injected with sodium citrate buffer alone. One week after the injections group 3 and 4 mice were place on a high-fat diet (HFD) after confirming hyperglycemia (glucose >= 14 mmol/l). HFD with 60% of fat (lard) source was purchased from ssniff GmbH, Germany (Catalog no. E15742-34). Anti IL-1β antibody (Ab) was kindly provided by Novartis, Basel, under a material transfer agreement (2015). Antibody was injected intraperitoneally once a week at 10 µg/g of mouse for the first two weeks and then at 5 µg/g of mouse until the end of the experiment (4 weeks). Saline injections were given in control mice (groups without antibody administration).

For inducing diabetes in B6 mice, 5-week-old mice were placed on HFD (D12331, Research Diets, New Brunswick, NJ) containing 58, 26 and 16% calories from fat, carbohydrate, and protein. In the 9th week, mice were injected with a single dose of STZ (i.p., 130 mg/kg of mouse) along anti IL-1β antibody at 10 µg/g of mouse for first two weeks and then at 5µg/g of mouse until the end of the experiment (4 weeks).

2.7.2. Measurement of blood glucose, serum Fetuin-A, urinary TNF-α, and urinary albumin levels

For measuring the fasting blood glucose, mice were fasted for 6 hrs in the morning and measurements were done using a Glucometer (Freestyle; Abbott Diabetes Care Inc., Alameda, CA) by taking a drop of blood from tail vein. Serum FetA and urinary TNF-α were measured using Duoset Elisa kit (R&D Systems) and mesoscale kit (Mesoscale Discovery, Rockville, MD), respectively, following the manufacturer's instructions. Urinary albumin levels were determined by employing a kit from Exocell (Philadelphia, USA) following the manufacturer's instructions.

2.8. Statistical analysis

All experiments were performed at least three times, and a representative experiment is shown. Data are expressed as means ± SD unless otherwise indicated. One way ANOVA was performed and for calculating significance of differences, Bonferroni post hoc test was employed. The prism 6 program was used for the analysis and differences were considered significant when P value was < 0.05.

3. RESULTS

3.1. Inflammatory response to FFAs and/or Fetuin-A in podocytes

3.1.1. Fetuin-A but not palmitic acid or oleic acid stimulate MCP-1 or KC secretion in podocyte

To investigate whether palmitic acid leads to an inflammatory response in podocytes, we measured MCP-1 and KC in the supernatant of podocytes after incubation with 75 µM palmitic acid for 12 hours. As shown in figures 3 and 4, palmitic acid did not stimulate the secretion of these cytokines compared to the control media with BSA not complexed to palmitic acid. As it has been suggested that FetA may be necessary for the inflammatory response elicited by palmitic acid (Pal et al., 2012), podocytes were incubated with FetA or FetA combined with palmitic acid (Figures 3 and 4). In contrast to palmitic acid, FetA significantly stimulated MCP-1 and KC secretion by 368 ± 57% ($p<0.001$) and by 1729± 135% ($p<0.001$), respectively, and the combination of FetA and palmitic acid led to a further increase of MCP-1 and KC by 229±28% ($p<0.001$) and by 194 ± 3% ($p<0.001$) compared to FetA alone.

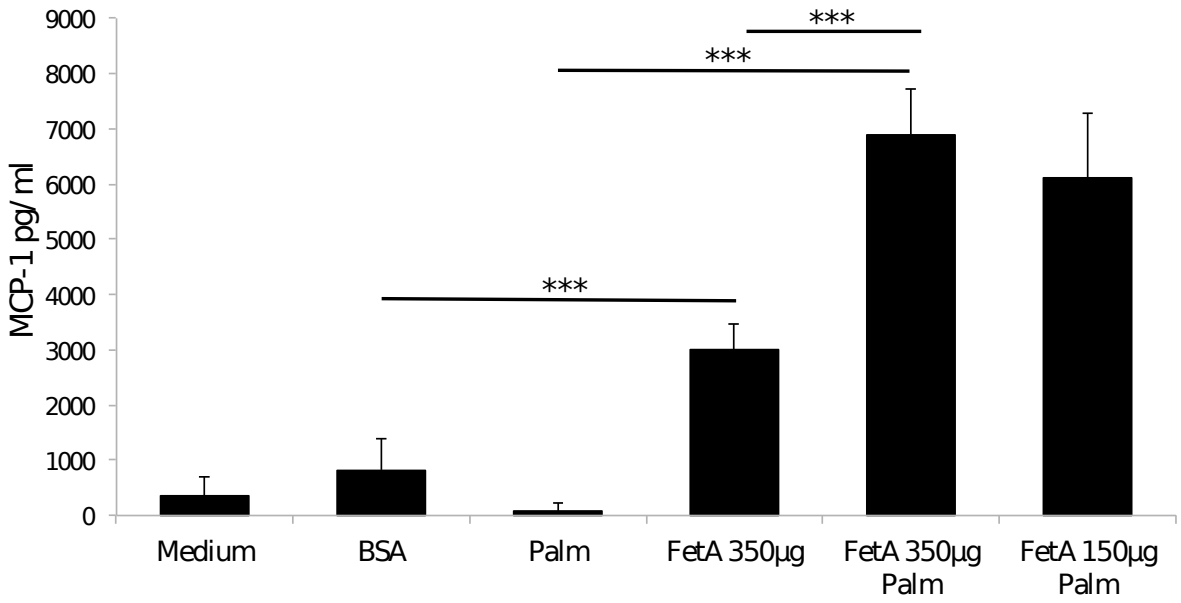

Figure 3: Murine Fetuin-A but not palmitic acid stimulates MCP-1 secretion in podocytes. MCP-1 release was quantified in podocytes treated with 75µM palmitic acid (or BSA control) in the presence or absence of 150-350 µg/ml bovine FetA. Bar graph represents mean ± SD of MCP-1 levels (in pg/mL) in the culture media at 12 hrs of treatment. (n = 6, ***p < 0.001).

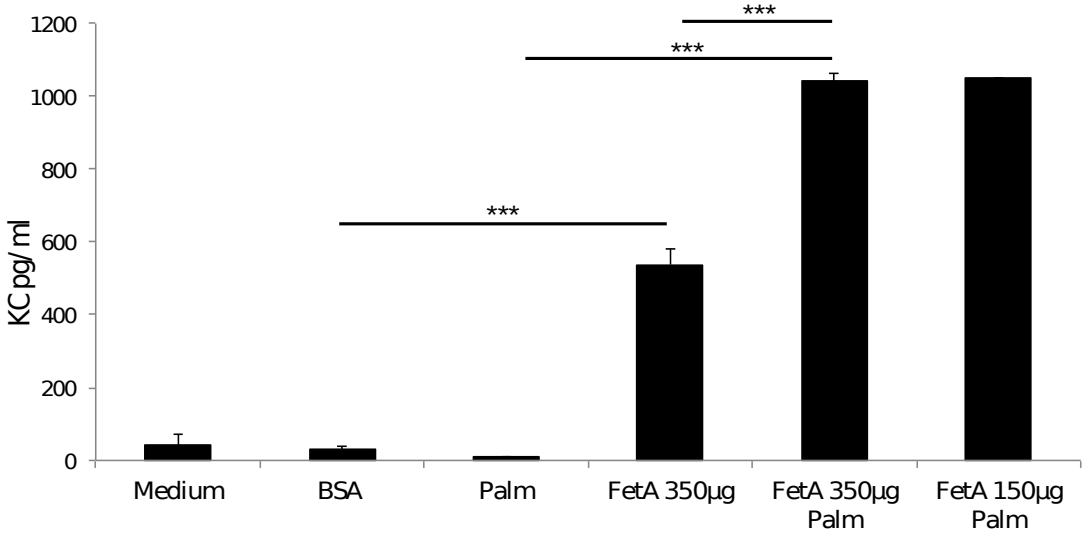

Figure 4: Murine Fetuin-A but not palmitic acid stimulates KC secretion in podocytes. KC release was quantified in podocytes treated with 75µM palmitic acid (or BSA control) in the presence or absence of 150-350 µg/ml bovine FetA. Bar graph represents mean ± SD of KC levels (in pg/mL) in the culture media at 12 hrs of treatment. (n = 6, ***p < 0.001).

As shown it Figure 5, similar results were obtain for oleic acid which was not able to stimulate MCP-1 secretion significantly, but FetA combined with oleic acid significantly increased MCP-1 by 173 ± 10% (p<0.001) compared to FetA alone.

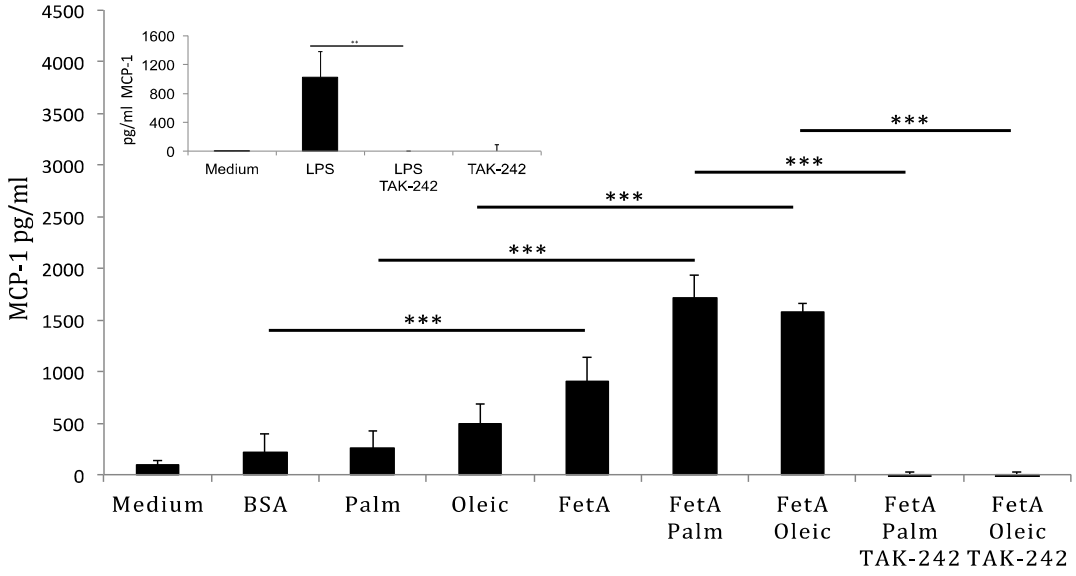

Figure 5: FetA alone or in combination with free fatty acids induces MCP-1, and the TLR4 blocker TAK-242 prevents chemokine induction. MCP-1 secretion was analyzed in podocytes treated with 75 µg/ml palmitic acid, 75 µg/ml oleic acid alone or in combination with 200 µg/ml FetA

in the presence or absence of TAK-242. Bar graph represents mean ± SD MCP-1 levels (in pg/mL) in the culture media at 16 hrs of treatment. TAK-242 was applied 1h prior to treatment (n = 4, ***p < 0.001). Insert: TAK-242 prevents LPS induced MCP-1 release. Podocytes were pretreated with TAK-242 (or vehicle) and subsequently incubated with 5 ng/ml LPS. Bar graph represents mean ± SD MCP-1 levels (in pg/mL) in the culture media at 16 hrs of treatment (n = 4, **p < 0.01).

As the proinflammatory response of FetA is reported to involve TLR4, we inhibited TLR4 signaling with TAK-242 (resatorvid), a specific small-molecule inhibitor of TLR4 signaling. TAK-242 completely prevented the increased MCP-1 secretion by the prototypic TLR ligand LPS (Figure 5, insert) as well as FetA in combination with palmitic or oleic acid (Figure 5).

3.1.2. Fetuin-A and LPS activates TLR4 in a dose dependent manner

To further investigate the potential role of FetA in stimulating the TLR-4 signaling pathway, I used a commercially available TLR4 reporter cell line. As shown in figure 6, increasing concentrations of FetA from 50 to 150 µg/ml dose dependently stimulated TLR4 similar to a logarithmic increase of lipopolysaccharide (LPS) concentrations.

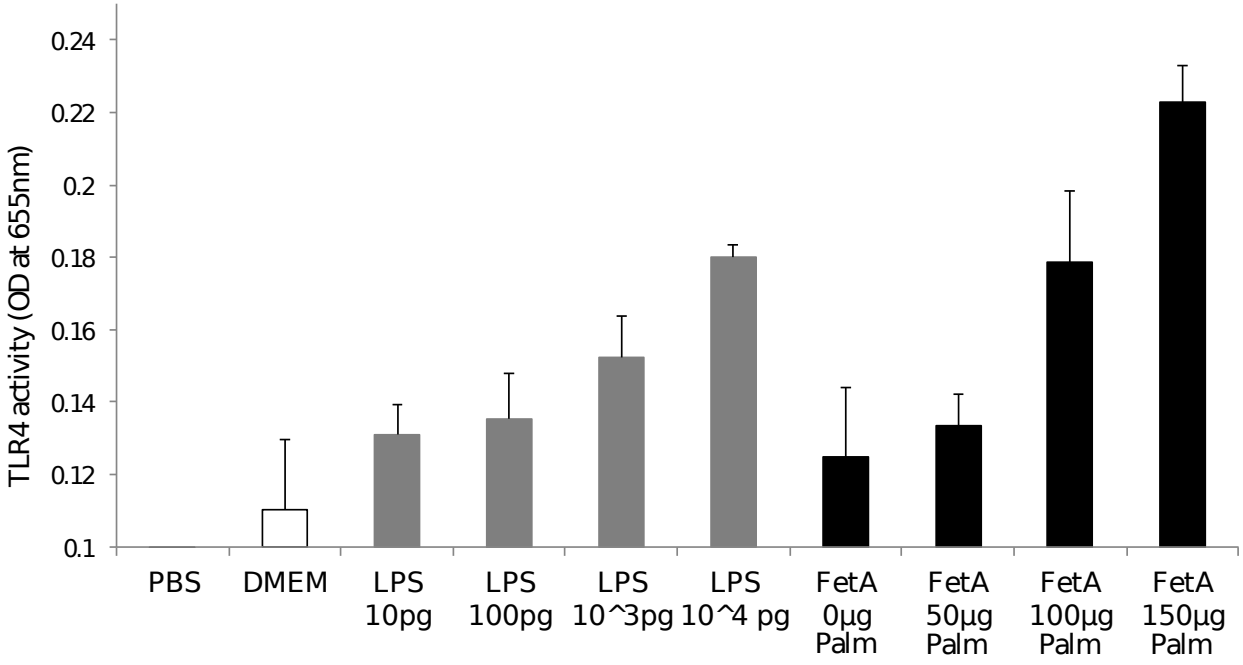

Figure 6: LPS and murine Fetuin-A activate TLR4 dose-dependently. TLR4 activity was measured in HEK-Blue™ hTLR4 cells treated with LPS (10 pg, 100 pg/ml, 10^3 pg/ml, 10^4 pg/ml) or FetA in the absence or presence of palmitic acid (0 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml) for 18h. Bar graph represents mean percentages ± SD of O.D. at 655nm (n = 3).

3.1.3. Fetuin-A aggravates palmitic acid induced podocyte death, and TLR4 inhibition attenuates palmitic acid induced podocyte death

Previously, we reported that palmitic acid dose- and time-dependently induces podocyte death (Sieber et al., 2010). To investigate whether FetA modulates cell death induced by palmitic acid, podocytes were treated with FetA alone or in combination with 200 µM palmitic acid for 48 hours. As shown in Figure 7, FetA had no effect on podocyte viability, but exacerbated palmitic acid induced podocyte death. Specifically, FetA further increased apoptotic podocytes (Annexin V single positive cells) by 20.6 ± 0.6% ($p < 0.05$). In this experiment, no further increase in necrotic (Annexin V, PI double positive cells) podocytes could be measured due to the disintegration of late necrotic podocytes, a well-known phenomenon reported previously by us (Sieber et al., 2010). Similarly, we observed that LPS had no effect on podocyte death, but increased palmitic acid induced apoptosis 28.5 ± 7.2% ($p < 0.001$) (Figure 8). TAK-242 could prevent the FetA induced increase in apoptotic podocytes (Figure 7) as well as the increase induced by LPS (Figure 8).

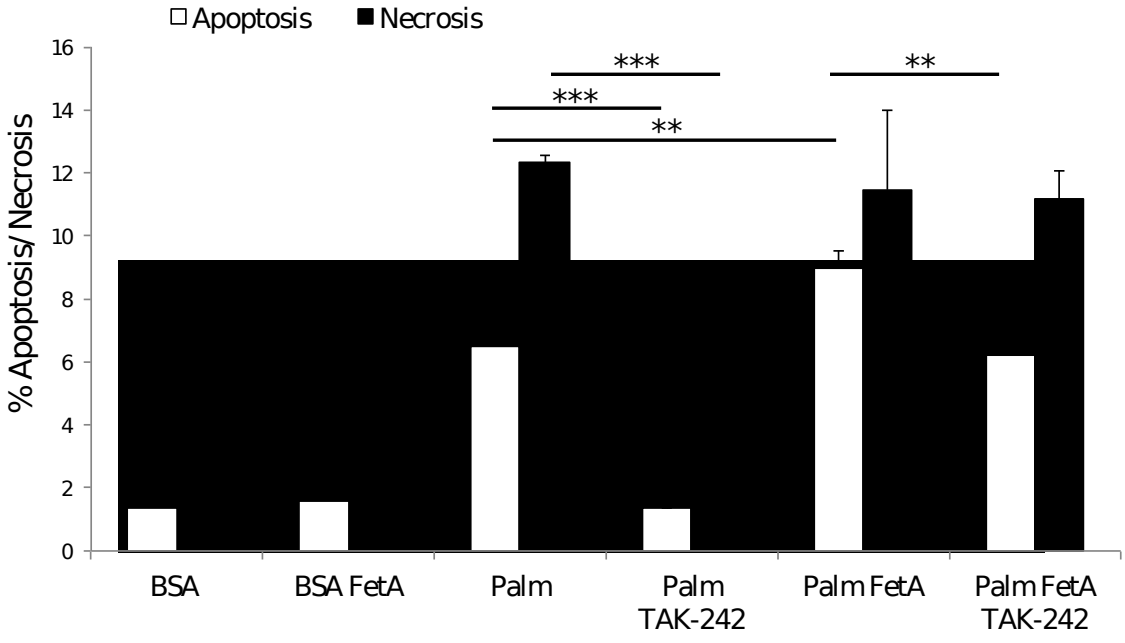

Figure 7: Bovine Fetuin-A aggravates palmitic acid-induced podocyte death, which can be attenuated by TLR4 inhibition. Podocytes were preincubated with 1 ng/ml TAK-242 for 1 h

and treated with 200 µM palmitic acid in the presence or absence of FetA (200 µg/ml) for 48 hrs. Bar graph represents the mean percentages ± SD of apoptotic and necrotic podocytes (n = 3, **p < 0.01, ***p < 0.001).

Of note, compared to podocytes treated with palmitic acid alone, the presence of TAK-242 decreased palmitic acid induced apoptosis by 79.3 ± 0.7% (p < 0.001) and necrosis by 65.3 ± 5% (p < 0.001) (Figure 7). This effect was unexpected and may indicate modulation of podocyte death by a constitutive activation of TLR4, which can be blocked by TAK-242.

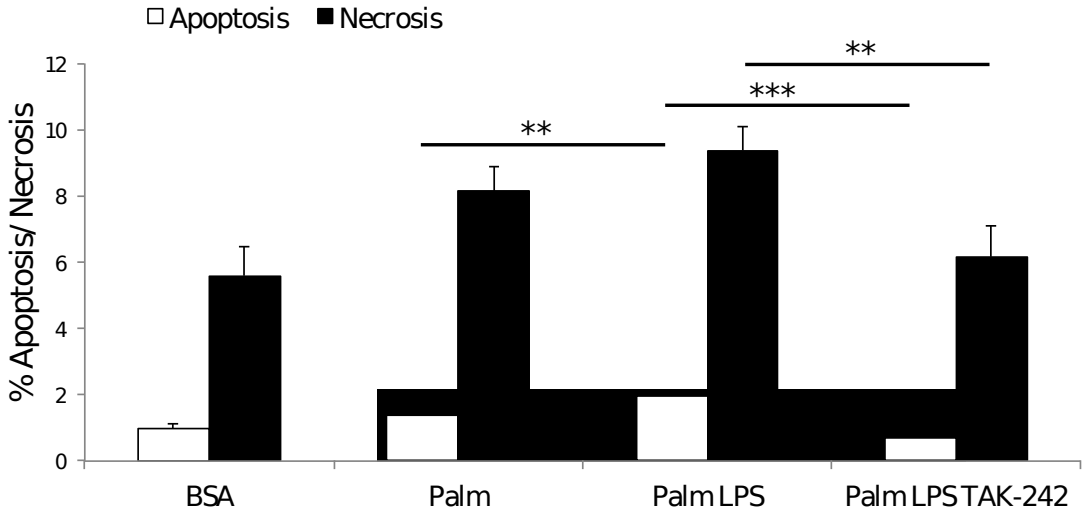

Figure 8: LPS aggravates palmitic acid-induced podocyte death, which can be attenuated by TLR4 inhibition.
Podocytes were preincubated with 1 ng/ml TAK-242 for 1 h and treated with 200 µM palmitic acid in the presence or absence of or LPS (5 ng/ml) for 48 hrs. Bar graph represents the mean percentages ± SD of apoptotic and necrotic podocytes (n = 3, **p < 0.01, ***p < 0.001).

3.1.4. IL-Ra and anti-1Lβ antibody attenuate FetA induced MCP-1 secretion in podocytes

The proinflammatory cytokine IL-1β is not only involved in the pathogenesis of experimental models of glomerulonephritis but also contributes to the development of diabetic nephropathy. To investigate whether IL-1β is involved in MCP-1 secretion induced by FetA and palmitic acid, I used a recombinant human IL-1Ra antagonist (anakinra) and a murinized neutralizing anti-IL-1β antibody. I treated podocytes with 200

µg/ml FetA alone or in combination with 75 µM palmitic acid for 16 hrs. FetA alone increased MCP-1 release by 227% ± 31 (p<0.001), and the combination of FetA and palmitic induced a further significant increase.

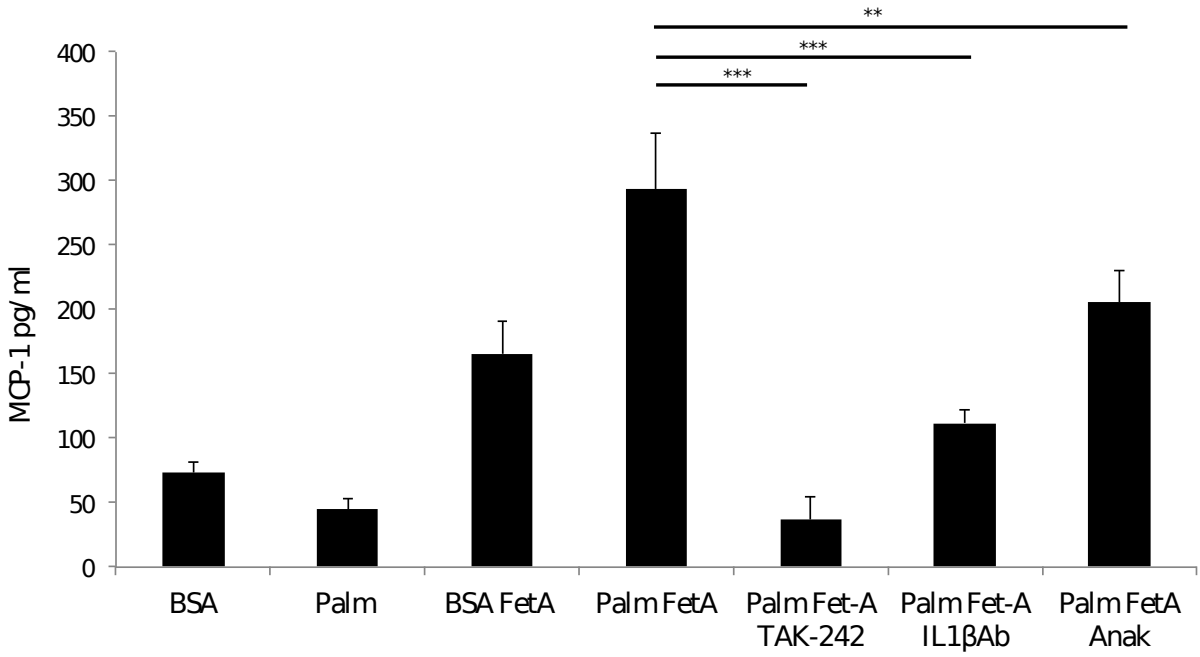

Figure 9: TLR4 inhibition and IL-1 neutralization prevent the MCP-1 release mediated by the combination of Fetuin-A and palmitic acid. Podocytes were preincubated with 1 ng/ml TAK-242, 1 µM anakinra or 3.3 µg/ml IL-1β anti-IL-1β antibody and subsequently treated with 75 µM palmitic acid alone or in combination with 200 µg/ml FetA. Bar graph represents mean ± SD of MCP-1 levels (in pg/mL) in the culture media at 16 hrs of treatment (n = 3, *p < 0.05, **p < 0.01, ***p < 0.001).

Preincubation with the TLR4 blocker TAK-242 (1 ng/ml) for an hour decreased MCP-1 release by 90% (p<0.001) compared to Fetuin A with palmitic acid. Similarly, the anti-IL-1β antibody reduced MCP-1 secretion by 53.4 ± 17% (p<0.001) and the IL-1Ra (anakinra) by 30 ± 8.8% (p<0.001) (Figure 9).

3.1.5. IL-1Ra and anti-IL-1β antibody attenuate Fetuin-A exacerbated palmitic acid induced podocyte death

To examine whether the injurious effect of FetA involves IL-1 signaling, podocytes were preincubated with anti-IL-1β or IL-1Ra for one hour before incubation with palmitic acid or palmitic acid and FetA for 48 hours. Both, the anti-Il-1β antibody and recombinant IL-1Ra significantly reduced apoptosis by 33.6 ± 6.2% ($p<0.05$), 30.2 ± 6.2% ($p<0.05$), and necrosis by 28.7 ± 4.7% ($p < 0.05$) and 31.9 ± 3.4% ($p<0.01$), respectively (Figure 10).

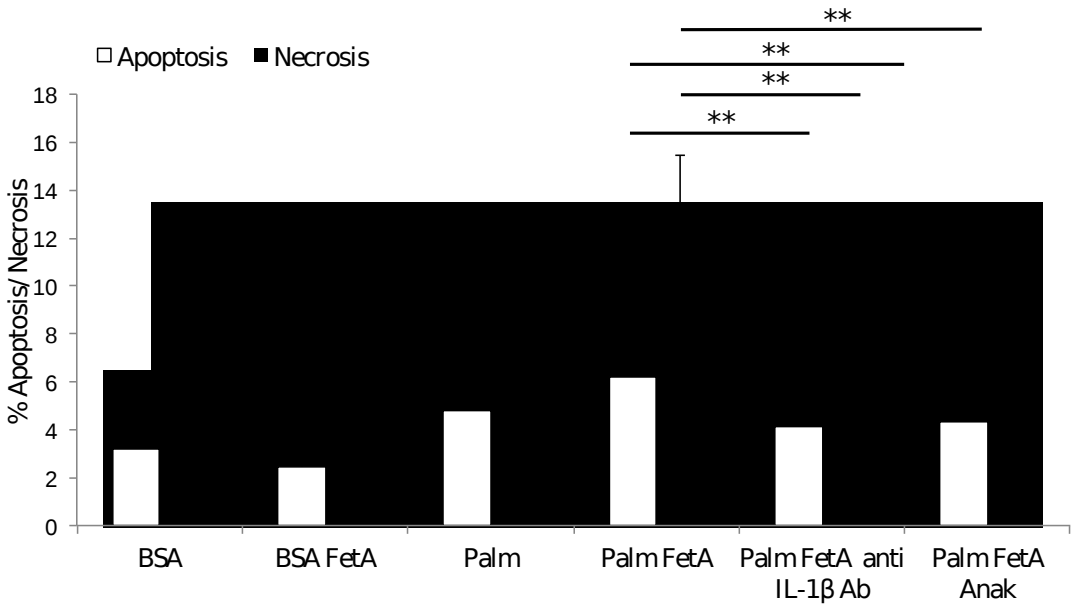

Figure 10: Bovine Fetuin-A exacerbates palmitic acid induced podocyte death, and TLR4 blockage or neutralizing IL-1ß is protective. Podocytes preincubated for 1 h with 3.3 µg/mL anti-IL-1β antibody or 1 µg/ml anakinra were treated with 200 µM palmitic acid alone or in combination with 200 µg/ml FetA for 48 hrs. Bar graph represents mean percentages ± SD of apoptotic and necrotic cells. (n = 3, ** $p < 0.01$).

3.1.6. Antagonizing IL-1 protects from palmitic acid induced podocyte death, and from podocyte death induced by the combination of LPS and palmitic acid

Similarly to the results shown in figure 10, IL-1Ra reduced the combined toxic effect of palmitic acid and LPS by a reduction in apoptosis of 44.5 ± 14.2% ($p < 0.01$) and necrosis of 23 ± 12.6% (NS) (Figure 11). Interestingly, IL-1Ra was also able to reduce palmitic acid induced apoptosis and necrosis (Figure 11) and for the anti-IL-1β antibody I could observed a reduction of palmitic acid induced apoptosis by 47 ± 10.5% (p

< 0.01) (Figure 12).

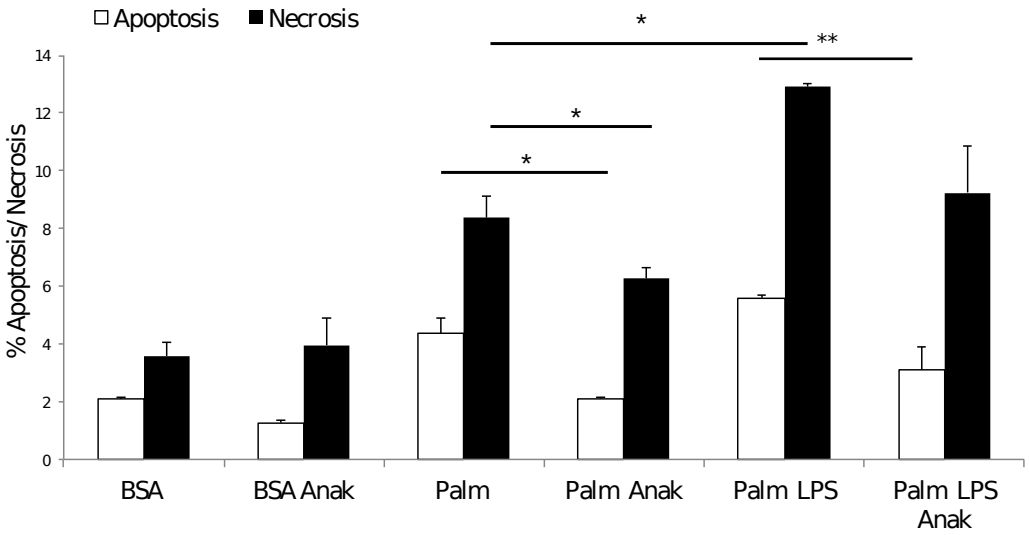

Figure 11: IL-1R antagonist protects from palmitic acid induced podocyte death, and from podocyte death induced by the combination of LPS and palmitic acid. Podocytes preincubated for 1 h with 1 µg/mL anakinra were treated with 200 µM palmitic acid alone or in combination 1 ng/ml LPS for 48h. Bar graph represent mean percentages ± SD of apoptotic and necrotic cells. (n = 3, * < 0.05, ** p < 0.01).

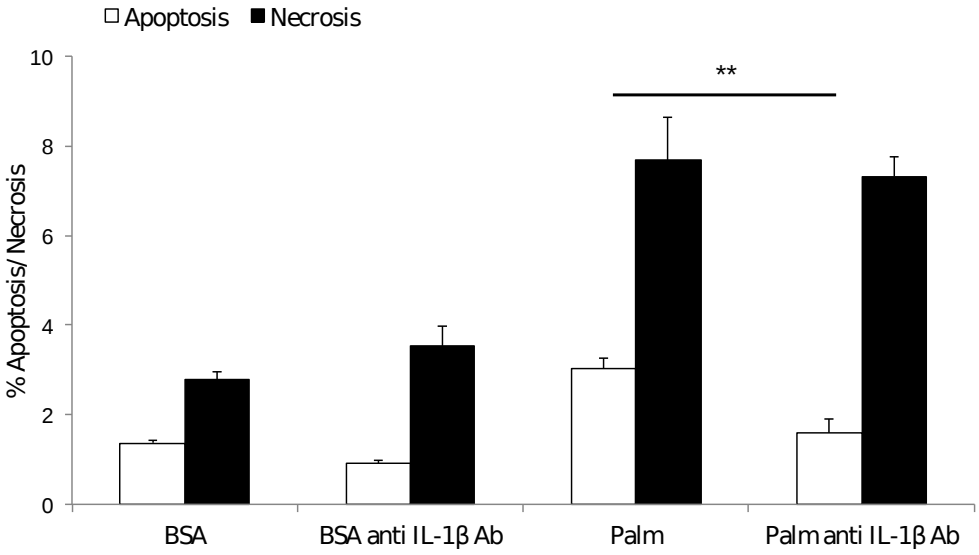

Figure 12: Anti IL-1ß Ab ameliorates podocyte death induced by palmitic acid. Podocytes preincubated for 1h with 3.3 µg/mL anti-IL-1β antibody and exposed to 200 µM palmitic acid for 48 hrs. Bar graph represents mean percentages ± SD of apoptosis and necrosis (n = 3, **p < 0.01).

3.1.7. Interleukin-1β in combination with palmitic acid exacerbates podocyte death

In the next step, podocytes were treated with either 200 µM palmitic acid or BSA (control) in the presence or absence of 5 ng/ml IL-1β for 48 hrs. The treatment with palmitic acid combined with IL-1β significantly accentuated the toxicity caused by palmitic acid, i.e. increases of necrosis and apoptosis by 190 ± 24% ($p < 0.001$) and 217 ± 35% ($p < 0.001$) were observed (Figure 13). The effect of IL-1β alone on podocyte death was minimal and did not reach statistical significance.

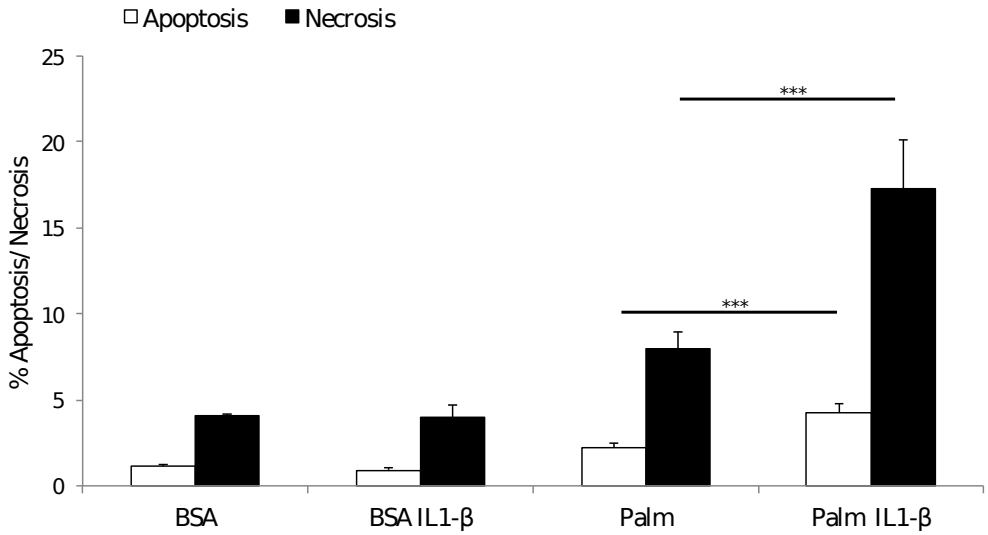

Figure13: IL-1ß aggravates podocyte death induced by palmitic acid. Podocytes were exposed to 200 µM palmitic acid in the presence or absence of 5 ng/ml IL-1β for 48 hrs. Bar graph represents mean percentages ± SD of apoptosis and necrosis (n = 3, ***$p < 0.001$).

3.2. Pilot experiment with anti-IL-1β antibody in diabetic mice

3.2.1. Anti IL-1β treatment reduces serum Fetuin-A levels in diabetic mice

To complement our *in vitro* findings we investigated the effect of the anti-IL-1β antibody in diabetic DBA/2J (DBA) mice.

Table 2 shows the characteristics of DBA/2J mice injected with STZ along with mouse IL-1β antibody or saline control. As indicated in table 2, average fasting blood glucose levels were 3 fold higher in diabetic mice than in control mice at the end of the experiment.

Table 2: Metabolic characteristics of DBA/2J mice experiment:

	Control + Saline	Control + anti IL-1β Ab	STZ (HFD)+ saline	STZ (HFD)+ anti IL-1β Ab
Age (weeks) at start of experiment	9	9	9	9
Weight at induction of Diabetes (g)	26.5 ± 0.96	26.5 ± 1.82	25.8 ± 1.53	25.7 ± 1.35
Weight at end of experiment (g)	33.3 ± 2.35	33.3 ± 2.85	24.8 ± 2.06	25.5 ± 1.67
Fasting Blood Glucose (mmol/l) at 10 day after STZ injection	5.5 ± 0.88	5.6 ± 0.27	11.2 ± 2.55	12.0 ± 2.48
Fasting glucose (mmol/l) at the end of experiment	5.8 ± 1.72	5.4 ± 0.86	17.4 ± 2.06	18.0 ± 2.04

Data are means ± SD (n = 6 mice in each group)

As shown in figure 14, serum FetA levels were significantly upregulated in diabetic mice (188 ± 16.4%, $p < 0.001$) compared to control mice. The

administration of the anti IL-1β antibody reduced the FetA levels by 63.3 ± 5% ($p < 0.001$).

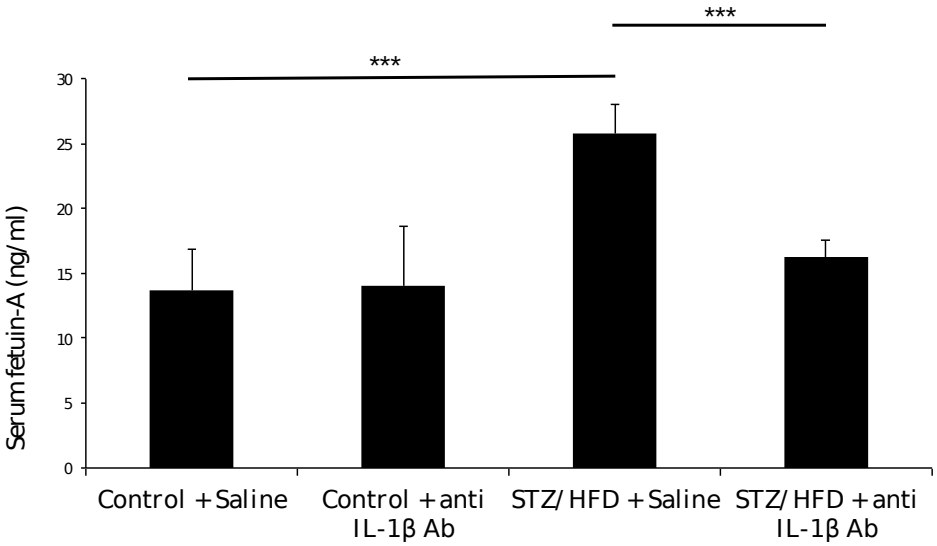

Figure 14: Anti-IL-1ß treatment reduces serum Fetuin-A in DBA/2J diabetic mice. Serum FetA was quantified in diabetic mice treated with an anti-IL-1β antibody (or vehicle) for 4 weeks (see method for experimental details). Bar graph represents serum FetA (ng/ml) levels ± SD in treated and untreated diabetic DBA/2J mice (n = 6, *** $p < 0.001$).

In another experiment, C57BL6/N were made diabetic by feeding a HFD for 4 weeks followed by an injection with a single dose of STZ at 130mg/kg of mouse. Table 3 shows the metabolic parameters of these mice. The fasting glucose levels were 1.9 fold higher in diabetic mice (HFD + STZ) compared to the control mice (chow diet + saline).

Table 3: Metabolic characteristics of B6 mice experiment:

	Control + Saline	Control + anti IL-1β Ab	HFD/STZ+ saline	HFD/STZ+ anti IL-1β Ab
Age (weeks) at start of experiment	9	9	9	9
Weight at induction of Diabetes or before STZ injection (g)	27.2 ± 0.93	27.2 ± 1.06	27.2 ± 1.66	27.2 ± 0.93
Weight at end of experiment (g)	31.5 ± 2.08	32.1 ± 2.42	28.2 ± 2.64	28.8 ± 2.07
Fasting glucose (mmol/l) at the end of experiment	5.5 ± 1.60	6.9 ± 1.26	10.39 ± 6.6	9.31 ± 2.50

Data are presented as arithmetic mean ± SD (n =6 mice in each group)

Treating the B6 with anti-IL-1β Ab significantly reduced serum FetA levels in diabetic mice (figure 15) by 21.3 ± 9.3% ($p < 0.05$).

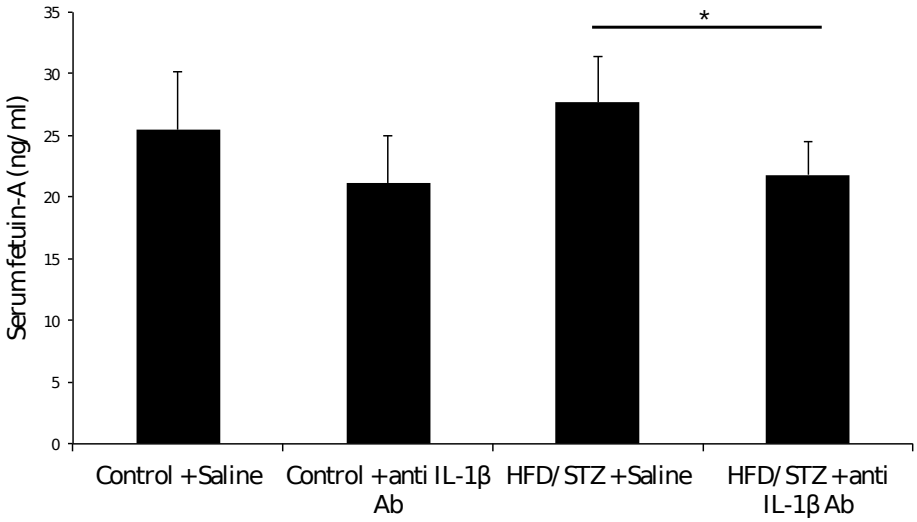

Figure 15: Anti-IL-1ß treatment reduces serum Fetuin-A in C57BL6/N diabetic mice. Serum FetA was quantified in diabetic mice treated with an anti-IL-1β antibody (or vehicle) for 4 weeks (see method for experimental details). Bar graph represents serum FetA (ng/ml) levels ± SD in treated and untreated diabetic B6 mice (n = 6, * p < 0.05).

3.2.2. Anti IL-1β treatment reduced urinary TNF-α levels in diabetic mice

As previous studies demonstrated that there is an increase in urinary TNF-α levels during the progression of albuminuria (Kalantarinia, Awad, & Siragy, 2003), we investigated whether treatment with the anti-IL-1β antibody affects urinary TNF-α in the model of diabetic DBA/2J mice. Figure 16 shows that diabetic mice showed increased TNF-α levels in the urine by 69.1 ± 18.5% ($p < 0.01$) compared to non-diabetic control mice. Treatment with the anti-IL-1β Ab significantly reduced TNF-α levels by 70.5 ± 2% ($p < 0.001$) compared to diabetic mice treated with saline.

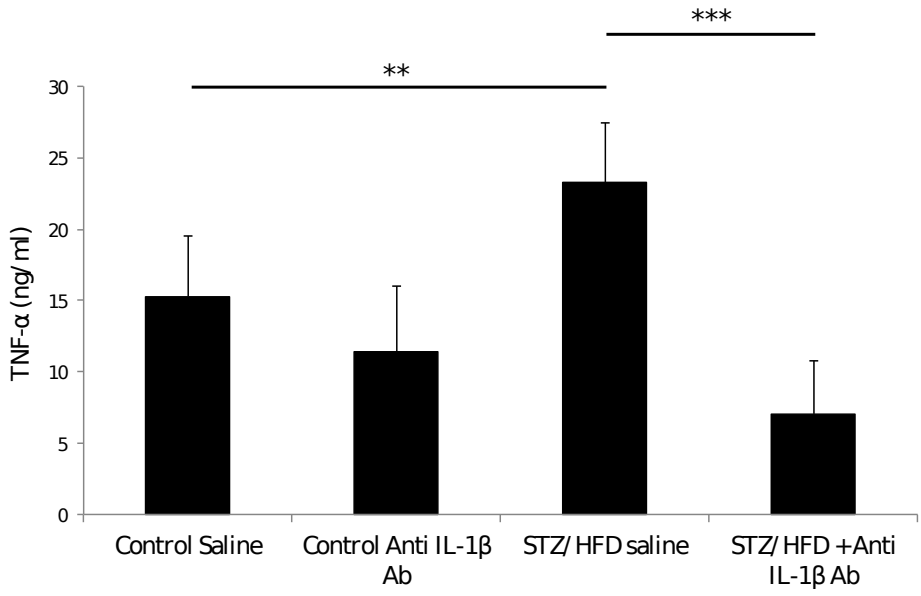

Figure 16: Anti-IL-1ß treatment reduces urinary TNF-alpha in diabetic DBA/2J mice. Urine TNF-α levels were quantified in diabetic mice treated with an anti-IL-1β antibody (or vehicle) for 4 weeks (see method for experimental details). Bar graph represents urine TNF-α levels (ng/ml) in treated and untreated diabetic DBA/2J mice (n=6; **p< 0.01, ***p<0. 001).

3.2.3. Short term anti-IL-1β treatment has no beneficial effect on urinary albumin levels in diabetic mice

To investigate if anti-IL-1β treatment affects urinary albumin levels in diabetic DBA/2J mice, albuminuria was measured. As shown in figure 17, albumin levels were significantly increased from control group to STZ group by 299.2 ± 74.2% ($p < 0.01$). However, there is no significant difference in albumin levels between treated group (STZ/HFD + anti-IL-1β Ab) and control (STZ/HFD + saline).

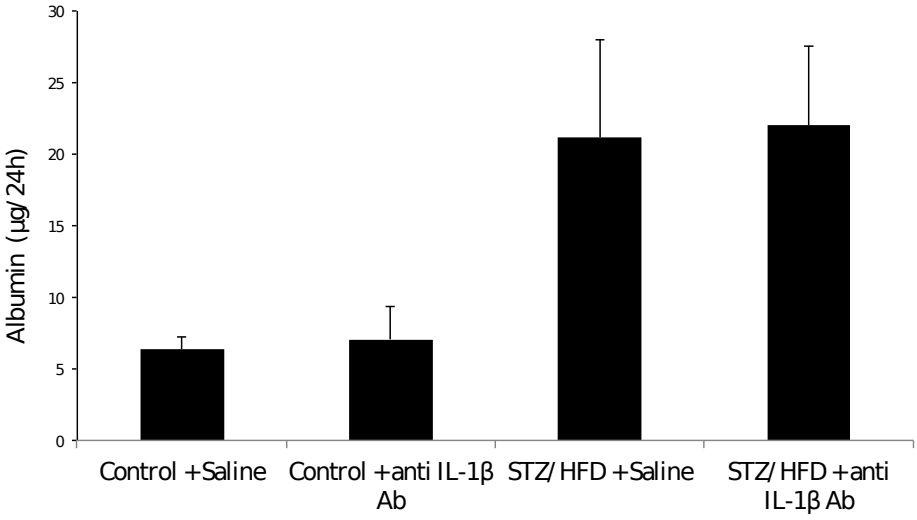

Figure 17: Anti-IL-1ß effect on urine albumin excretion in diabetic DBA/2J mice. Urine was quantified in diabetic mice treated with an anti-IL-1β antibody (or vehicle) for 4 weeks (see method for experimental details). After 10 days of injection, STZ groups were placed on high fat diet (HFD) and control groups were maintained on chow diet for four weeks. Anti IL-1β antibody was administered at 10 µg/g of mice for first two weeks and then concentration of antibody was maintained at 5 µg/g of mice. Bar graph represents mean (SD) of urinary albumin levels collected for 24 hrs (n=6, ** $p<0.01$)

4. DISCUSSION

In the present study, we demonstrate that FetA aggravates palmitic acid induced podocyte death. This effect is associated with an inflammatory response and involves IL-1β.

Several lines of evidence suggest that the deleterious effect of FetA involves TLR4 signaling. Specifically, using genetically engineered HEK-Blue hTLR4 cells as a reporter system FetA led to a dose-dependent signal by increasing FetA from 50 to 150 ug/ml. A similar dose response was observed with the prototypical TLR4 ligand LPS. In contrast to FetA, the dose of LPS was increased logarithmically from 10 up to 10^4 pg/ml. This suggests that the mechanism of FetA on TLR4 may differ from LPS. To minimize interference with any residual endotoxin contamination, FetA was cleaned with polymyxin B and residual endotoxin concentrations were below 0.1 EU/ml. As the source of murine FetA tried up during the study we were forced to switch to bovine FetA (experiments performed with murine FetA are limited to Fig. 5 and 6). For an unexplained reason, the results with the HEK-Blue-hTLR4 reporter cell line could not be confirmed with bovine FetA (data not shown). A species difference may account for this difference. Importantly, in podocytes both bovine and murine FetA significantly stimulated MCP-1 release (Fig. 3, 5 and 9) which was further increased by the FFAs palmitic or oleic acid. Consistent with the reporter assay data, the specific TLR4 inhibitor TAK-242 (Matsunaga, Tsuchimori, Matsumoto, & Ii, 2011) prevented the MCP-1 release in podocytes (Fig. 5 and 9) and our data suggests that the observed proinflammatory response involves TLR4 signaling.

Interestingly and in contrast to previous studies using other cells including pancreatic β-cells (Boni-Schnetzler et al., 2009; Lee, Sohn, Rhee, & Hwang, 2001; Shi et al., 2006), no stimulation of MCP-1 or KC could be observed in podocytes incubated with palmitic acid alone although palmitic acid together with FetA exacerbated the inflammatory response. This may be explained by inherent differences of specific cell types. Alternatively, most

cell culture studies are performed with 10% fetal bovine serum (FBS), which contains about 20 mg/ml FetA (Pal et al., 2012). Previously it has been suggested that FetA, FFAs, and TLR4 build a ternary complex (Pal et al., 2012). Alternatively, palmitic acid may augment TLR4 signaling by enhancing the concentration of the sphingolipid ceramide (Jin et al., 2013) which is known to affect cell signaling at multiple levels, e.g. by modulation of raft composition and assembly of the TLR4 signaling complex (Plociennikowska, Hromada-Judycka, Borzecka, & Kwiatkowska, 2015). As FetA is well known as a carrier protein (Cayatte et al., 1990) an intriguing hypothesis is that it acts as a sink for FFAs and endotoxin thereby potentially increasing their local concentration or availability. Clearly, more experiments are needed to clarify the exact interplay between FetA, FFAs, and TLR4.

Of note, both FetA and LPS had no effect on podocyte survival but exacerbated palmitic acid induced podocyte death and this could be partially prevented by the TLR4 inhibitor TAK-242 (Fig. 5 and 6). The finding that LPS exacerbated palmitic acid induced podocyte death may at least in part explain albuminuria in septic patients (Basu et al., 2010) as bacterial endotoxins and FFAs are increased in this setting (Nogueira et al., 2008).

Remarkably and unexpectedly, TAK-242 dramatically reduced palmitic acid induced podocyte death. As FetA, BSA, and palmitic acid complexed to BSA were cleaned with polymyxin B, stimulation of TLR4 by any residual endotoxin concentration is unlikely, but cannot be excluded. If so, such a residual endotoxin concentration would be in the lower range of healthy human blood donors, reported to be between 1.00 – 0.01 EU/ml (Nadhazi, Takats, Offenmuller, & Bertok, 2002). Alternatively, the protective effect of TAK-242 on palmitic acid induced podocyte death could be explained by a constitutively active TLR4 in podocytes. Although TAK-242 binds specifically to an intracellular domain of TLR4 (Matsunaga et al., 2011) we cannot rule out a protective off-target effect. Excluding the later possibility, our data are entirely consistent with and extend previous reports indicating that TLR4 signaling can adversely affect the kidney and contributes to DN (J. Wada & Makino, 2016).

An interesting outcome of the current study was that inhibition of IL-1 signaling by anakinra, a recombinant human IL-1Ra, or a murinized anti-IL-1β antibody could attenuate the inflammatory response elicited by FetA and palmitic acid. Similarly, anakinra and the anti-IL-1β antibody attenuated podocyte death induced by combined treatment with palmitic acid and FetA or by palmitic acid alone. This clearly indicates that palmitic acid induced podocyte death is partially mediated by IL-1β. Interestingly, podocyte death could not be induced by IL-1β alone as it was previously reported in pancreatic beta-cells (Maedler et al., 2004), however IL-1β was able to exacerbate palmitic acid induced podocyte death. Together this indicates that the proapoptotic effect of IL-1β depends on a second hit elicited by palmitic acid or by the combination of palmitic acid and FetA in podocytes.

In a pilot study we investigated the effect of a murinized anti-IL-1β antibody in insulinopenic DBA/2J mice (STZ model) put on a high fat diet. Although the experiment had to be terminated prematurely after five weeks and no beneficial effect on the threefold increase in albuminuria could be observed, treatment with the anti-IL-1β antibody largely prevented the substantial increase in the serum FetA concentration. This observation could be confirmed in a second pilot experiment using a slightly different protocol using C57B6/N mice. Together with our in vitro data, this observation is tempting to hypothesize that the lower FetA concentrations may serve a surrogate marker and may predict longtime protection by the anti-IL-1β antibody against DN. In this context, a community-based study in China is particularly interesting as people in the highest FetA tertile had a 2-fold increased risk for developing albuminuria over a follow up period of four years (Lv et al., 2016). Moreover, treatment with the anti-IL-1β antibody suppressed the increase in urinary TNF-α levels observed in diabetic mice. This indicates a protective effect of an anti-IL-1β therapy as urinary TNF-α is a risk factor for albuminuria (Kalantarinia et al., 2003) and TNF-α is reported to induce podocyte death (Ryu, Mulay, Miosge, Gross, & Anders, 2012; Tejada et al., 2008). Using a different approach a recent study demonstrated the likely important role of IL-1 signaling and of the inflammasome in the pathogenesis of DN. In this

study mice deficient in Nlrp3 (nucleotide-binding domain and leucine-rich repeat pyrin 3 domain) in non-myeloid-derived cells were protected from DN (Shahzad et al., 2015). Furthermore anakinra protected from or even reverse DN in different mouse models (Shahzad et al., 2015). Therefore, prolonged treatment with the anti-IL-1β antibody is likely to show a protective effect beyond decreasing serum FetA and urinary TNF-α levels and further in vivo studies are needed to investigate this therapeutic approach for the prevention and treatment of DN.

5. CONCLUSION

The present study emphasizes the critical role of inflammation for the survival of podocytes. The results reported indicate that pro-inflammatory cytokines and chemokines downstream of TLR4 mediate palmitic acid induce podocyte death.
The results suggest that FetA alone or in combination with FFAs stimulate pro-inflammatory cytokines and chemokines in podocytes through TLR4 activation and in this way modulate podocyte survival. More specifically, LPS or FetA alone failed to induce podocyte death but consistently exacerbated palmitic acid induced podocyte death and inhibition of TLR4 prevented MCP-1 and KC release in podocytes and reduced podocyte death induced by palmitic acid alone or in combination with FetA. Moreover, the data presented here indicate that IL-1β plays a key role in palmitic acid induced podocyte death, as an anti-IL-1β antibody or the recombinant IL-1Ra antagonist anakinra attenuated podocyte death induced by palmitic acid alone or in combination with FetA.

In vivo, therapy with a murinized anti-IL-1β antibody for 5 weeks in STZ treated DBA/2J mice was not able to protect from albuminuria, but prevented an increase in serum FetA concentrations and considerably decreased TNFα expression in urine, calling for prolonged studies to clarify this therapeutic approach.

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